Candida albicans mitochondrial dynamics during a yeast to hyphal transition
by Scott D Kobayashi

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University

Abstract:
Candida albicans mitochondria (mt) undergo morphological changes during cell cycle events, however, the genetic basis for this change is not well understood. Electrophoresis of C. albicans total cellular DNA yields several intense ethidium bromide-stained moderately sized (>2.5 kilobase pairs) MspI (C/CGG) fragments. These apparent repeat units are shown to be mt in origin. MspI digests of mtDNA were cloned into the ClaI site of pBluescript II. Four different mt-specific clones were chosen as probes to determine relative mt genome copy number in C. albicans wild-type and mutant strains grown in a serum-containing medium. The cloned fragments were radiolabeled and used to probe dot blots of total cellular DNA digests. Hybridization dot intensities were compared against similarly labeled C. albicans single-copy actin DNA to obtain an estimate of mt genome copy number. Stationary phase yeast cells contained approximately 3.4 mt genomes, as compared to 4.8 in 3 hr. germ tubes. These results indicate that mt genome copy number increases over time during a yeast to hyphal morphogenesis. Hyphae-deficient mutants also yielded a higher number of mt genomes than wild-type strains. In addition to mt genome copy number changes, we detected changes in specific activities of both succinate dehydrogenase and cytochrome oxidase. Comparison studies using cellular homogenates and crude mt fractions indicated that individual mt undergo changes in enzyme activity prior to mt division and growth. These findings imply that mt processes are important in the yeast to hyphal transition.

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APPROVAL

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Scott D. Kobayashi

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Candida albicans mitochondria (mt) undergo morphological changes during cell cycle events, however, the genetic basis for this change is not well understood. Electrophoresis of *C. albicans* total cellular DNA yields several intense ethidium bromide-stained moderately sized (>2.5 kilobase pairs) *Msp*I (C/CGG) fragments. These apparent repeat units are shown to be mt in origin. *Msp*I digests of mtDNA were cloned into the *Cla*I site of pBluescript II. Four different mt-specific clones were chosen as probes to determine relative mt genome copy number in *C. albicans* wild-type and mutant strains grown in a serum-containing medium. The cloned fragments were radiolabeled and used to probe dot blots of total cellular DNA digests. Hybridization dot intensities were compared against similarly labeled *C. albicans* single-copy actin DNA to obtain an estimate of mt genome copy number. Stationary phase yeast cells contained approximately 3.4 mt genomes, as compared to 4.8 in 3 hr germ tubes. These results indicate that mt genome copy number increases over time during a yeast to hyphal morphogenesis. Hyphae-deficient mutants also yielded a higher number of mt genomes than wild-type strains. In addition to mt genome copy number changes, we detected changes in specific activities of both succinate dehydrogenase and cytochrome oxidase. Comparison studies using cellular homogenates and crude mt fractions indicated that individual mt undergo changes in enzyme activity prior to mt division and growth. These findings imply that mt processes are important in the yeast to hyphal transition.

Chapter 1

INTRODUCTION

Increasing numbers of immunocompromised individuals have resulted in a rising incidence of disease attributed to opportunistic pathogens, including those caused by fungal agents. Candidiasis, an infection caused by fungal organisms in the *Candida* genus, often occurs as an opportunistic infection in immunocompromised individuals. Importantly, along with progressive disseminated histoplasmosis, cryptococcosis, and invasive aspergillosis, *Candida* infections are considered diagnostic hallmarks of acquired immunodeficiency syndrome (AIDS) (227,273). In addition to AIDS, improved life-sustaining technologies and aggressive anticancer therapy have contributed to the increasing incidence of candidiasis over the past two decades (99). In fact, *Candida* species have become the fourth leading cause of all nosocomial blood stream and urinary tract infections in U.S. hospitals (105).

The major etiological agent of candidiasis is *Candida albicans,* which constitutes 76% of clinical *Candida* isolates (272). *C. albicans* is a member of the human normal flora and can be isolated from the mouth (51), gastrointestinal tract (68), vagina (233), and skin (175). *C. albicans* has a broad clinical spectrum that can vary from superficial mucosal lesions (332) to life-threatening systemic or disseminated disease (174). The diverse clinical spectrum of infections, the limited number of cost-effective and safe antifungal agents (358), and the lack of ideal typing and diagnostic methods for *C. albicans* (166) have made it increasingly difficult to control candidiasis. Although the

number of available antifungal agents has increased over the past several years, the emergence of drug resistant strains and the relative toxicity of current antifungal therapy validate research aimed at discovering novel drug targets. Of particular interest is the mitochondrial organelle. Several therapeutic agents directed against the mitochondria of other human pathogens are effective (91), thus substantiating the utility of antimitochondrial therapeutics and the rationale behind mitochondrial research.

The mitochondria of *C. albicans* have been poorly characterized to date. The majority of information on mitochondrial structure, function, and genetics in fungi has been gained from research on several non-pathogenic organisms, including *Saccharomyces cerevisiae* (382). My dissertation has focused on several unresolved features of the *C. albicans* mitochondrial genome. One feature of mitochondrial organelles in general, is that they are dynamic with respect to both size and number throughout the cell cycle and in response to environmental conditions (21,153,354). One unique feature of *C. albicans* is that it is a polymorphic fungus which exhibits three different morphologies both *in vivo* and *in vitro*: yeast, pseudohyphae, and hyphae. Although several systemic fungal pathogens undergo morphogenesis, they are usually found as yeasts in humans (191). *C. albicans* is capable of existing in either form in the host and there has been much speculation on the role of hyphae in pathogenesis. Hyphal formation *in vitro*, as discussed below, can be induced by various growth conditions. I chose the yeast-to-hyphal transition to study differences in the mitochondrial genome copy number and changes in mitochondrial activity. The remainder of this introduction gives an overview of the current level of understanding of *C. albicans* mitochondria. I

will discuss clinical aspects of candidiasis, morphogenesis of *C. albicans*, and mitochondrial function and genetics as they pertain to my research topic.

Clinical Aspects of Candidiasis

In 1970, the National Nosocomial Infections Surveillance (NNIS) system was established to obtain data from U.S. hospitals regarding the type and incidence of nosocomial infections by use of standardized procedures (174). According to the NNIS, *Candida* species accounted for 72.1% of all fungal nosocomial infections between 1980 and 1990 (20). *C. albicans* is clearly the leading cause of candidiasis, representing 76% of all clinical *Candida* isolates (272). However, recent reports suggest that shifts have occurred in the distribution, and thus importance of non-*albicans* species of *Candida* (274,282,369). *C. glabrata*, *C. tropicalis*, *C. krusei,* and *C. lusitaniae* have become more frequently reported in association with infection (25). The mortality attributable to invasive *Candida* infections is quite impressive with crude mortality rates estimated at 50 to 60% in total (43,198), and as high as 73 to 90% in bone marrow transplant patients (237). In hopes of reducing the severity of disease, many epidemiological surveys have been undertaken to specify at-risk populations (7,174).

Predisposing Factors to Candidiasis

Multiple factors have been ascribed to increased susceptibility to *Candida* infections and have been thoroughly reviewed in the literature (259). As mentioned previously, *Candida* species can cause mucocutaneous and disseminated forms of candidiasis. Disseminated disease is usually associated with compromised immunity.

Important factors that induce immunosuppression in the host include aggressive chemotherapy (184) and corticosteroid treatment (198). Neutropenia, which may be induced by drugs or disease, is also a predisposing factor to disseminated candidiasis (364). Prolonged administration of antibacterial agents tends to promote the overgrowth of *Candida* sp. and can lead to a clinical manifestation of disease (190). Additional risk factors are those that provide a nidus of infection such as indwelling venous catheters (24,29) or invasive surgical procedures (43). Underlying disease is another risk factor as demonstrated by the high incidence of candidemia in patients with acute lymphocytic leukemia (307).

Diagnosis of Candidiasis

Early initiation of antifungal therapy is critical in reducing the mortality of disseminated candidiasis (7,154). The determination of systemic candidiasis is not a trivial consideration and in fact, has been problematic. Traditional diagnostic blood cultures (90) and antibody-based diagnostic schemes (98) are often times negative in candidiasis patients. In many cases, these two methods yield negative results despite histopathologic evidence of *Candida* invasion (163). In one study, 56% of necropsy proven cases were negative by blood culture (76). In addition, there is a poor correlation between systemic infection and candidemia. Candidemia does not always lead to systemic disease (360) and experimental animal models often show low levels of fungemia (167). Newer approaches for early and rapid detection of invasive candidiasis include monitoring the serum and/or urine levels of *Candida* cell-wall antigens, such as mannan/mannoproteins (251,279) and enolase (356), and *Candida* metabolites, such as

D-arabinitol (213). PCR-based methods to detect *Candida* DNA are also in the early stages of development (180). Clinical assessment of these various diagnostic tests has been performed, and though promising compared to blood culture, they tend to lack specificity and/or sensitivity (229).

Anticandidal Drug Therapy

Management and treatment of candidiasis rely primarily on the use of antifungal agents, both prophylactically and therapeutically. The current repertoire of antifungal drugs with systemic activity includes: amphotericin B, the azoles, and 5-fluorocytosine. Unfortunately, these drugs have several limitations including toxicity to the host and/or a rapid development of resistance by *Candida* (185). Amphotericin B is directed against ergosterol, a major component of the fungal plasma membrane. Ergosterol contributes to a variety of important cellular functions required for cellular growth and division (152,351). Amphotericin B is inserted into fungal membranes and functions by disruption of the membrane integrity (192). Although amphotericin B is historically the drug of choice for systemic candidiasis and other systemic fungal infections, this drug has important clinical limitations. Renal dysfunction and infusion-related toxicities are commonly associated with high dosage therapy (350), and drug-resistant strains of *Candida* species are increasingly reported (256).

5-fluorocytosine (5-FC) is another antifungal agent that is effective against *Candida*. 5-FC is transported into the cell by a cytosine permease and metabolized to form 5-fluorodeoxyuridine monophosphate and 5-fluorouridine triphosphate (192). The net result is disruption of both DNA and protein synthesis, respectively. Although quite

effective in susceptible yeast strains, the use of 5-FC is limited by a rapid development of resistance, particularly when used as a single agent (104). Natural resistance to 5-FC is also a concern because it occurs in up to 20% of *C. albicans* strains tested (335). 5-FC is most frequently used as adjunctive therapy with amphotericin B, however, toxicity of 5-FC remains a concern. The use of combination therapy has increased toxic effects due to 5-FC from less than 5% to 15-30% of patients (104). Conversely, the use of combination therapy reduces the optimal dose and toxicity levels of amphotericin B.

The azole antifungals have become important therapeutic agents for the treatment of candidiasis, owing to their relative safety, ease of delivery, and overall effectiveness (22,137). The azoles prevent the demethylation of lanosterol, an intermediate in ergosterol synthesis and are generally fungistatic agents (177). The azoles most commonly used for the treatment of disseminated candidiasis include: ketoconazole, fluconazole, and itraconazole (185). Fluconazole has become widely used for the treatment of AIDS-associated oropharyngeal candidiasis (9) and to prevent and treat candidiasis in neutropenic patients (217). The rise in popularity of fluconzole is likely due to a high oral bioavailability (69). The prophylactic use of fluconazole has also been shown to decrease the incidence of invasive candidiasis in patients receiving chemotherapy and bone marrow transplantation (126,322). Fluconazole is also effective against vaginal candidiasis in single-dose therapy (325). Itraconazole and voriconazole are also promising candidates, but their clinical usefulness is still under investigation. Unfortunately, concomitant with the widespread usage of azole antifungals has been the

emergence of drug resistant strains. *C. krusei* and *C. glabrata* are intrinsically resistant to fluconazole (260).

The development of fluconazole resistance is a complex issue. Many studies report increased fungal resistance in patients receiving high total cumulative doses of fluconazole (283), long-term therapy (238), and recent exposure to fluconazole (281). Conversely, one study revealed that fluconazole resistance can occur without prior exposure and furthermore, *C. albicans* developed resistance in several patients receiving large doses of fluconazole (355). In that study, *C. albicans* resistance to single dose treatment with fluconazole is also reported.

The limitations of current antifungal therapy have renewed interest not only in the development of more effective agents, but also in the identification of novel therapeutic targets. Ergosterol has been the primary target for drugs directed against fungal cell membranes. Recently, a new generation of antifungals directed at another essential membrane component, sphingolipids (5), have been discovered. Aureobasidin A is an inhibitor of sphingolipid synthesis. In a mouse model of systemic candidiasis, aureobasidin A is more effective than either amphotericin B or fluconazole (342). It was suggested that aureobasidin A is a strong inhibitor of expression of the ABC transporter/efflux pump, CDR2 (141). Increased expression of the *CDR* genes is correlated with azole resistance in *Candida* (296), which implies that aureobasidin A should be a good candidate for conjunctive therapy with fluconazole.

Morphogenesis

> "Few fields of biological science based on so simple an observation, can have generated such a confused and contradictory literature as that of dimorphism in *Candida albicans*"- F.C. Odds (259).

C. albicans is a polymorphic fungus, as it can exist as a simple yeast form or undergo morphogenesis and produce filaments in the form of pseudohyphae and/or hyphae. Pseudohyphae are characterized by constrictions at septal junctions upon polar bud formation and tend to form in chains, whereas hyphae form parallel walls during apical extension and develop true septa. All forms are usually present in clinical lesions, which has led to considerable speculation and experimentation on the virulence role of hyphae in the pathogenesis of candidiasis (71).

The formation of hyphae in *C. albicans* occurs in response to several different *in vitro* mechanisms of induction. The properties and conditions supporting *in vitro* hyphal growth have been reviewed (259). In general, environmental stimuli such as temperature, pH, serum, CO_2, and nutrition, may induce a yeast-to-hyphal transition *in vitro* (248). The mechanisms behind hyphal production are enigmatic, which has led to the controversy surrounding hyphae as a virulence factor. Based on the current level of understanding of *C. albicans* morphogenesis, the role of hyphae in pathogenesis is inconclusive.

Cell Biology and Physiology of Morphogenesis

Insights into the process of morphogenesis in *C. albicans*, as well as many other filamentous fungi, have surprisingly come from information gained from wild-type cells

and not from mutants. In *C. albicans*, early processes in germinating cells are very similar to normal yeast production. Initiation of germination under appropriate growth conditions leads to an increase in the rate of volume growth of the mycelium identical to that of a budding yeast cell (148). The surface areas to volume ratios, however, are quite different with a much larger surface area in germlings. Implied in this finding is that initial hyphal growth is accompanied by the production of a thinner cell wall, which is in agreement with ultrastuctural analysis on *C. albicans* germ tubes (58,178). Cell-wall biosynthesis and cell extension occurs primarily at the tapered apical region (125,133). In growing hyphae, approximately 90% of wall extension occurs in the hyphal tip, whereas in budding yeasts only 70% occurs at the apex of the bud (331). Cytoplasmic content in growing hyphae remains constant, undergoes a steep rise in pH (334) and is accompanied by a migration of protoplasm from the parent cell to the hyphal apex (134). Initial hyphal elongation proceeds at a linear rate (45), but only the apical cells are metabolically active. Intercalary compartments remain uninucleate (136), largely vacuolated (134), and are arrested in the G1 phase of the cell cycle (136). Prior to the formation of secondary germ tubes and branches in mature *C. albicans* hyphae, cytoplasm is regenerated and growth proceeds at a logarithmic rate (135,148).

The induction of germination and hyphal production requires specific changes in the architecture of the cell wall. The exoskeleton of the fungal wall is composed of a complex network of polymers that imparts rigidity and determines the cell shape. The cell wall in *C. albicans* is composed of mannoproteins (20-30%), β1,3-linked glucans (25-35%), β1,6-linked glucans (35-45%), protein (5-15%), lipid (2-5%), and chitin (.6-

2.7%) (340). Cellular growth involves the formation of newly synthesized wall components in conjunction with the degradation or rearrangement of existing structures. The localization of secretory vesicles to the apical tip is required for hyphal elongation. The vesicles are highly refractive and can be seen by use of brightfield microscopy (38,157). Transport of the secretory vesicles from Golgi equivalents in *C. albicans*, requires actin microfilaments (379). Initiation of germination is accompanied by increased levels of actin transcripts (78,210,268). The role of cytoplasmic microtubules in morphogenesis is less clear. In one study, specific inhibition of microtuble formation by nocodazole in pre-germinated cells led to normal hyphal production (379). In another study, treatment of yeast cells with the anti-microtubule drug benomyl inhibited the formation of germ tubes and changed the distribution of actin filaments (3). These data indicate that *C. albicans* microtubules may be more important for the initiation of germination than for the production of mature hyphae. Microtubules and associated motor proteins, kinesins and dyneins, are involved in organelle localization and hyphal growth in several other filamentous fungi (88,142,156,372).

Information is limited on mechanisms of cell wall degradation and synthesis in *C. albicans*, but changes involving specific polysaccharides during germ tube formation have been reported. For example, the cell wall of germ tubes contains proportionately more β–1,3-linked glucans than either yeasts or mature hyphae (127). A three- to five-fold increase in cell wall chitin occurs immediately following germ tube formation (56). Similarly, studies on mRNA levels of chitin synthase genes during germination showed increased expression that peaked 1-2 hours after induction (61,248,339). Disruption of a

hyphal-specific chitin synthase gene (*CSH2*) did not effect germination or virulence (130). The transcription of chitinase also demonstrates significant differences between yeast and hyphae. Transcription levels of two chitinase genes are greater during yeast cell growth versus hyphal growth (234). This implies that the restructuring of chitin is more important in yeast cell growth than in hyphal cells.

Several reports have implicated a role for sterols in hyphal formation. Free sterol content progressively increases during germination (240), and the total sterol content in mycelial lipids greatly exceeds that of yeasts (115). Sterol biosynthesis inhibitors, including the majority of azole antifungals, have also been shown to negatively effect germination (52,316). Several cell wall proteins are associated with morphogenesis in *C. albicans* (table 2), however, their role in hyphal production is unclear.

Hyphae as a Virulence Factor

A virulence factor, simply defined, is any factor produced by a fungus that increases virulence in the host. Virulence in *C. albicans* is a complex issue and appears to be the result of multiple factors (71). Many investigators have focused on *C. albicans* morphogenesis as a putative virulence characteristic. However, the evidence for hyphae as a virulence factor is unclear. For example, there is a general lack of agreement on the predominating form in either colonization or disease (259). In fact, both yeast and filamentous forms are usually present at sites of infection (51). These observations do not preclude the role of hyphae in pathogenesis. As described below, comparative studies with yeast and hyphal cells indicate that differences beyond cell type exist. In

many cases hyphae demonstrate increased adherence and invasive properties, which are characteristics involved in the pathogenesis of *C. albicans* (258).

Adherence. The ability of *C. albicans* to adhere to various surfaces and tissue/cell types has been intensely investigated and reviewed (51,71,259,270). Hyphal forms more readily adhere to human epithelia (194,326) and endothelia (289) than yeast cells. This hyphal property may be due to increased hydrophobicity as compared to yeast cells (254). Hydrophobic yeast cells show greater binding to epithelial cells than hydrophilic yeasts (93).

Invasion. Hyphae or the production of hyphae have been implicated in the invasion of host tissue. Several investigators have reported increased numbers of hyphal cells involved in both epithelial and endothelial penetration (96,158,289). Migration of *C. albicans* across the endothelial cell layer was proposed as a prerequisite for multi-organ involvement in disseminated candidiasis (196,381). If this hypothesis is correct, then increased hyphal penetration of endothelium suggests a mechanism for dissemination of *C. albicans*. There is little evidence to substantiate this conclusion.

Thigmotropism. Ordered hyphal growth in *C. albicans* is a characteristic that may facilitate tissue invasion. Two independent reports have demonstrated a helical growth pattern of *Candida* hyphae on a solid surface (179,313). In addition, *C. albicans* hyphae have been shown to grow by contact sensing, or thigmotropism (131,253,314). In these studies, hyphal extension proceeded along grooves and through pores on a solid membrane support. This property may allow *C. albicans* hyphae to penetrate some

tissues by following surface discontinuities and microscopic lesions. There is little evidence that demonstrates thigmotropism *in vivo*, although parallel hyphal penetration into intracellular spaces has been observed by ultrastructural analysis of oral infections (277) and vaginal tissue (261).

Extracellular Enzymes. Tissue/cellular damage is associated with the production of hyphae and is proposed to be enzyme-mediated (14). Both hyphal and yeast forms of *C. albicans* are capable of producing secreted aspartyl proteinases (SAP), which may participate in tissue invasion (246). SAP genes are differentially expressed between yeast and hyphal forms. A multigene family with at least nine known members (*SAP1* to *SAP9*) is encoded by *Candida* (235). *SAPs 4,5*, and *6* are expressed only in germ tubes in media containing serum as a source of nitrogen (160,363). Gene disruption of these three related products yielded a strain with attenuated virulence in both guinea pig and mouse models of systemic candidiasis (295). Germination in the same triple deletion mutant was severely impaired. The significance of these data is unclear as it was not determined whether attenuated virulence was due to Sap enzyme deficiencies or abnormal hyphal production. The role of individual Sap isoenzymes in the virulence of *C. albicans* has not been clearly established. As discussed later, gene disruption experiments of putative virulence factors in *C. albicans* are often difficult to interpret.

Increased levels of another *Candida* putative virulence factor, extracellular phospholipase, are also associated with enhanced hyphal production (168). In this study, the levels of phospholipase production in several blood isolates of *C. albicans* were measured. These isolates were further analyzed for their ability to germinate and were

tested in a mouse model of disseminated candidiasis. A correlation was observed between increased phospholipase production, the ability to germinate, and increased mortality. Phospholipase, acid phosphatase and glucosaminidase activities were concentrated at the bud site of yeasts and the apical tips of germ tubes (351). The localization of these enzymes to regions of cellular growth most likely represents a role in structural changes required for cellular elongation and replication. This would explain why a correlation between increased phospholipase production and the ability to germinate was observed (168).

Genetics of Morphogenesis in *C. albicans*

Features of *C. albicans* that confounds genetic studies are that this fungus is diploid or polyploid and apparently lacks a sexual state (262). This property has not hindered genomic plasticity in this organism. Genomic plasticity in *C. albicans* is perhaps best demonstrated by extensive karyotypic polymorphism (291,292). Seven chromosomes have been ascribed to the *C. albicans* genome and each can vary in size as evidenced by pulsed-field electrophoretic analysis (220,221). The genetic basis of karyotype variation is unknown.

Historically, molecular biology and classical genetic analysis in this fungus have been hampered by the diploid state. The creation of mutants to address specific virulence factors (i.e., hyphal formation) relied upon non-specific mutagens and natural variants (table 1). Differences in virulence were observed between mutants and wild-type strains in mouse models of candidiasis (71). However, a major caveat of using these mutants in virulence studies is that they lack genetic characterization. *C. albicans* molecular

methods are available that facilitate the creation of specific genetic mutations. Complementation (129), gene disruption (204) and transformation systems (189) have all been described and can be used to create genetically defined *C. albicans* mutants.

Table 1. *Candida albicans* hyphal mutants of non-defined specificity

Mutant	Characteristics	Virulence/model	Reference
1. B311V6	Non-hyphal variant	Attenuated/ vaginitis	(326,362)
2. hOG301	Nitrosoguanidine -hyphae only	Non-pathogenic/ systemic	(159,310)
3. CA2	Echinocandin resistant non-hyphal variant	Attenuated/ vaginitis Attenuated/ systemic	(77) (293)
4. MM2002	Antibody selected non-hyphal variant	Virulent/ systemic	(293)

Saccharomyces cerevisiae As a Model to Understand *C. albicans* Morphogenesis

In efforts to determine whether the ability to form hyphae is important for virulence, several investigators have used *S. cerevisiae* as a model in which to identify *C. albicans* genes that may either enhance or suppress hyphal formation. This approach is based on the assumption that a similar mechanism of hyphal induction is shared between these two organisms. Diploid strains of *S. cerevisiae* undergo a yeast-to-pseudohyphal transition when nitrogen starved (121). In *Saccharomyces*, this transition depends on factors that participate in the mitogen-activated protein kinase (MAPK) pathway, including Ste7p, Ste11p, Ste12p and Ste20p (215). *Saccharomyces* mutants deficient in

any or a combination of these signal transduction gene products were defective in both mating and filamentous growth. Although *C. albicans* does not possess a complete mating pathway, this fungus has genetic elements that complement several mating defects in *Saccharomyces* (65,197,211,212,214,222,319,320). Sequential disruption (double-allelic knockout) of any of these mating gene homologs in *C. albicans* resulted in mutants with the inability to form hyphae under certain *in vitro* growth conditions (197,211,212,214). One fundamental difference between *S. cerevisiae* and *C. albicans* was that wild-type strains and gene-disrupted mutants of the latter formed hyphae in response to serum, whereas wild-type strains and mutants of *S. cerevisiae* did not. This observation implies important genetic differences in regulation, control and expression of filamentation between these two fungi.

The results of homologous gene studies yielded data that indicate further differences between *C. albicans* and *S. cerevisiae* filamentation pathways. Tup1, a general transcription repressor in *Saccharomyces* (187), participates in filamentation in both *S. cerevisiae* and *C. albicans* (39). Whereas the mutant *C. albicans* strain produced filaments on all media, the analogous mutant of *Saccharomyces* had a markedly reduced ability to produce pseudohyphae in a nitrogen-deprived medium. The point is, disruption of the *TUP1* gene yielded opposite effects in *C. albicans* as compared to *S. cerevisiae*. Although *C. albicans* shares many gene homologs with *S. cerevisiae*, the *TUP1* studies indicate that there are important differences in morphogenesis of these fungi.

Development of Agerminative Mutants

Efforts to develop agerminative *C. albicans* mutants have prompted investigation into the genetic basis of the residual filament formation exhibited by *Candida* strains deficient in MAPK gene homologs of *S. cerevisiae*. As mentioned above, whereas these *C. albicans* mutants were non-filamentous on serum-free media, they still formed hyphae in response to serum (197,211,214). Recently, in independent studies, Lo, et al. (216) and Stoldt, et al. (336) demonstrated that another putative transcriptional regulator, Efg1p, plays a role in hyphal formation. *EFG1* is a *C. albicans* gene homolog of *PHD1*, a *S. cerevisiae* pseudohyphal regulator (120). Either deletion of the gene in *C. albicans* (216) or reduced expression under an inducible promoter (336) yielded a pseudohyphal phenotype, rather than hyphae, in response to serum. Disruption of both *EFG1* and a MAPK gene homolog (*CPH1*) resulted in a *C. albicans* mutant (HLC54) that was incapable of hyphal formation under several different conditions, including a serum-containing medium (216). This finding is provocative in that it may provide insight into potential molecular switch mechanisms for morphogenesis. Unfortunately, the specific regulatory functions of the putative transcription factors Efg1p and Cph1p are unknown. If these factors affect multiple genes, the inability of a double mutant to form hyphae may be only one of several defects.

In attempts to address the question of whether the ability to form hyphae is a virulence factor, Lo et al. (216) designed experiments to test the pathogenicity of the HLC54 double mutant in a mouse model of disseminated candidiasis. The results of survival data indicated that HLC54 was less virulent than both wild-type *C. albicans* and

mutants lacking only one of the two transcription factors (Cph1p or Efg1p). On the basis of these data, the authors concluded that the ability of *C. albicans* to switch from yeast to the filamentous form was required for virulence. However, the results of their virulence studies on the heterozygous mutants, and discussion by the authors, support the suggestion that Cph1p and Efg1p transcription factors affect multiple genes. The *EFG1/efg1* heterozygotes, which are capable of normal hyphal production *in vitro* (20% serum), were less virulent than the *EFG1/EFG1* homozygote. This indicates that *EFG1* may participate in a function that is not linked to cellular morphogenesis, but that it influences virulence nevertheless. As an alternative explanation, the *EFG1/efg1* heterozygote may not produce normal hyphae *in vivo*, but this was not determined. In agreement with the alternative explanation, Stoldt, et al. (336) showed an abnormal yeast/pseudohyphal form at a low *EFG1* expression level. This indicates that in addition to defective hyphal production, the yeast forms may not have been normal. This point is further demonstrated by studies on the gene encoding CaCla4p, a putative serine/threonine protein kinase (212) similar to Ste20p. *C. albicans* strains deficient in CaCla4p produced truncated germ tubes in the presence of serum and showed reduced virulence in mice. However, yeast cells produced in this strain were often multibudded and multinucleated (212). This behavior of yeasts during cellular division indicates a defect in cytokinesis and is not typical of wild-type yeast cells. Deletion of another MAPK gene in *C. albicans*, *MKC1*, has also been linked to deficiencies in cell integrity and attenuated virulence (85), and not to hyphal production. Although the Lo, et al. (216) mutants approximate *in vitro* growth rates of the wild-type strain, this criterion does not

preclude important yeast cell wall alterations that could account for the reduced virulence of the double mutant. Lo, et al. (216) concluded that the "switch" from yeast to hyphal morphogenesis, and not necessarily the form of the fungus, is important for virulence. Conversely, the *EFG1/efg1* heterozygote underwent the switch, but were less virulent (216), and others found that *HST7* and *CST20* deletion mutants produced hyphae *in vivo*, but were less virulent than the wild-type (211). Furthermore, deletion of a gene coding for an integrin-like protein of *C. albicans* (Int1p) yielded very similar results to *HST7* and *CST20* disruption (111). The *int1/int1* mutant exhibited reduced hyphal production under several *in vitro* growth conditions and was less virulent than the wild-type strain. This homozygous mutant was capable of normal hyphal production when grown in serum-containing media. This indicates that the morphological switch is not an independent virulence factor.

The interpretation of *in vivo* experiments on virulence of the HLC54 mutant was further complicated by the choice of control strains. In the genetic analysis of the mutant, Efg1p function was confirmed by transformation of HLC54 with a functional *EFG1* gene, and subsequent restoration (strain HLC84) of the original filamentous phenotype. This critical revertant control was not used, however, in the mouse virulence studies.

Although hyphal growth is generally regarded as an important virulence trait of *C. albicans*, mutants expressing predominantly filamentous phenotypes are not more virulent than wild-type strains. In fact, the previously mentioned *TUP1* deletion mutants were less virulent in a mouse model of disseminated candidiasis (39). In addition to *TUP1*, the disruption of a serine/threonine kinase gene, *SNF1*, yielded an enhanced

hyphal phenotype (271). In *S. cerevisiae*, Snf1p interacts with the Tup1p repressor complex in glucose repression (378) and the RAS-cyclic AMP signal transduction pathway (347). *C. albicans SNF1* deletion mutants did not demonstrate increased virulence (271).

As pointed out by Lo et al. (216), the defined genetics of *S. cerevisiae* have been useful in providing insights into genetic aspects of morphogenesis of *C. albicans*. As we indicate here, there are limitations to conclusions that can be drawn from comparisons between these two species. In the past, the rationale for use of *Saccharomyces* as a model system to study the genetics of *C. albicans* was based primarily on the difficulties of working with the *Candida* diploid genome. Molecular techniques are now available for use in *C. albicans* that specifically address the complex genetic system of this organism. For example, methods for sequential gene disruption in *S. cerevisiae* (4) have been adapted for use in *C. albicans* (101). Importantly, such techniques allow studies to be done directly on *C. albicans*.

Conclusions

The search for the genetic basis of polymorphism in *C. albicans* has led to important recent discoveries concerning the basic molecular biology of this organism (table 2), including the initial characterization of putative transcription factors that affect hyphal formation. Several *S. cerevisiae* gene homologs are clearly involved in *C. albicans* morphogenesis. These elegant molecular studies, although fascinating, have not resolved the issue of hyphae formation and virulence in *C. albicans*. The multitude of significant differences between *C. albicans* and *S. cerevisiae*, including pathogenicity,

warrants direct studies on *C. albicans*. Revealing the unique features of the *C. albicans* genome will provide information about how this microorganism is able both to colonize and cause disease in humans.

Table 2. Morphogenesis associated genes in *Candida albicans*

Gene	Product/ Function	Deletion Phenotype	Virulence	Reference
1. *ECE1*	H specific antigen	Normal	N.D.	(33)
2. *PHR1/ PHR2*	pH-regulated glycoprotein	Defective Y and H	Attenuated	(116,247,297)
3. *PRA1*	pH-regulated H antigen	Defective H	N.D.	(39)
4. *HYR1*	Cell wall glycoprotein	Normal	N.D.	(17)
5. *CaRSR1*	Ras-related protein	Defective Y and H	Attenuated	(373)
6. *KEX2*	proprotein convertase	Defective H	N.D.	(252)
7. *PKC1*	protein kinase C	Defective Y and H	N.D.	(269)
8. *SNF1*	ser/thr prot. kinase	increased hyphae	Normal	(271)
9. *RBF1*	transcription factor?	hyphae only	N.D.	(169,170)
10. *MKC1*	MAPK	Defective Y and H	Attenuated	(85)
11. *INT1*	integrin-like protein	Defective H	Attenuated	(110,111)
12. *HST7*	MAPK	Defective H	N.D.	(211)
13. *CPH1*	MAPK	Defective H	Attenuated	(216,222)
14. *CST20*	MAPK	Defective H	Attenuated	(65,211,212)
15. *EFG1*	transcription factor?	Defective H	Attenuated	(216,336)
16. *TUP1*	transcription repressor	hyphae only	Attenuated	(39)

Mitochondria

As mentioned previously, the central topic of this thesis concerns *C. albicans* mitochondrial genome copy number changes during a yeast to hyphal transition. An understanding of the importance of mitochondria in the biology of *C. albicans* necessitates a review of knowledge gained from non-*Candida* systems.

Mitochondria are highly variable organelles in regard to size, shape, chemical composition and function. Mitochondria primarily serve as a site for respiration and oxidative phosphorylation, however several other functions have been attributed to these diverse organelles. They also synthesize lipids, heme, amino acids, nucleotides, and they mediate the intracellular homeostasis of inorganic ions (348). Although mitochondria contain an independently replicating genome, distinct from that of the nucleus, the majority of mitochondrial proteins are encoded by nuclear genes and are transported to the organelle (374). Studies on petite mutants in *S. cerevisiae*, deficient in respiration and mitochondrial DNA, indicate that function of an intact mitochondrial genome is required for the development of a respiration-competent organelle (16).

Structure

Mitochondria are membrane bound organelles consisting of an outer and inner membrane. Both membranes are composed primarily of proteins and lipids. Phosphatidyl choline, phosphatidyl ethanolamine, and cardiolipin represent the majority of mitochondrial lipids (143). Detailed lipid analysis has revealed differences between the outer and inner membrane. The outer membrane contains ergosterol whereas the

inner membrane has a higher level of cardiolipin and no ergosterol (173). The differences in protein composition of these membranes are attributed to the presence of functional enzyme components. The outer membrane contains proteins involved in the transport of macromolecules and lipid biosynthesis (305). The inner membrane contains proteins required for oxidative phosphorylation including the four respiratory complexes and the ATP synthetase (305). The inner matrix contains the mitochondrial genome and protein synthesis machinery (139).

Biogenesis/Protein Import

Mitochondria proliferate by growth and division of pre-existing organelles (374). During cellular division, the faithful segregation of mitochondria and mitochondrial DNA into daughter cells is essential for cell viability (16). The process of mitochondrial inheritance requires both biogenesis of membranes and cytoskeletal involvement during cell division. Protein import into mitochondria is the major mechanism of mitochondrial biogenesis (299). Most cytoplasmic proteins destined for transport into the mitochondrial matrix are synthesized with N-terminal amphiphilic targeting sequences (19). The loosely folded precursor protein binds to the mitochondrial surface, a process mediated by mitochondrial surface receptors (249). Protein import into the matrix space requires the function of the mitochondrial ATPase, hsp70 chaperonin (155) and the outer membrane transporter ISP42 (18,353). Hsp70 acts in conjunction with the Tim44 subunit of the inner membrane import complex, and the nucleotide exchange factor mGrpE (183,300). Once the protein has reached the matrix, its targeting sequence is removed by

a series of matrix enzymes (MAS1, MAS2, and hsp60) and the mature polypeptide is folded into its native conformation (113,306).

Mitochondrial Behavior

The behavior of fungal organelles during cellular growth and division has been studied extensively. Controversies exist with respect to the number of mitochondria per yeast cell and their morphology. Early descriptions range from numerous ovoid mitochondria to a single branched mitochondrion, based on electron microscopic imaging (17,153,348,373). Evidence for the presence of a single mitochondrion was obtained by reconstruction of serial sections of entire *S. cerevisiae* yeast cells (153). Further mitochondrial analysis has revealed that yeast mitochondria are dynamic in both structure and number in response to environmental changes (193,333,354), including anaerobic growth (73). In addition to *S. cerevisiae*, serial section electron microscopic reconstructions have been performed on both *Schizosaccharomyces pombe* and *C. albicans* (182,343), indicating that mitochondria size and number in these fungi are also dynamic.

Three-dimensional reconstruction studies from multiple serial sections of whole *C. albicans* cells demonstrated that the mitochondria are dynamic during both cell division and germ tube formation (343). In this study, mitochondrial behavior in three isolates of *C. albicans* was compared at different stages of the cell cycle. The progression of the cell cycle was correlated with the extent of bud emergence and spindle development in the nucleus. Mitochondrial fusion and division events were dependent on the stage in the cell cycle and not on the growth or increase in volume of mitochondria.

The mitochondria fused into a single giant organelle upon bud formation and fragmented during mitosis. Coalescence occurred again prior to cytokinesis and fragmented shortly thereafter. The ratio of organelle volume to total cell volume remained relatively constant. The estimated numbers of mitochondria varied between 1-4 in strain NUM678 and 2-10 in strain IAM4966, indicating that there are strain to strain differences. Similarly, mitochondrial behavior during morphogenesis demonstrated the formation of a giant mitochondrion prior to germ tube formation, which fragmented during mitosis. Mitochondrial numbers in germinating cells varied between 1 and 7 in strain IAM4966. The average percentage of cell volume occupied by mitochondria was between 6 and 13% which is similar to data reported for the yeasts *Pityrosporum ovale* (186) and *S. cerevisiae* (140).

The dynamic behavior of mitochondria in both *C. albicans* yeast and hyphae has also been observed by fluorescence microscopy (13,171). Mitochondria were fluorescently stained with 2-(4-dimethlaminostyryl)-1-methylpyridinium iodide (DASPMI), which preferentially stains mitochondria based on the electron potential of the membrane (30). In contrast with data obtained from reconstruction methods, Ito-Kuwa et al., (171) reported the presence of a single branched mitochondrion that divided only upon cytokinesis. In addition, there were no cell cycle-related morphological changes of mitochondria which is in direct agreement with studies on *C. utilis* and *C. tropicalis* (75). The authors concluded that the form and number of mitochondria of *C. albicans* are primarily influenced by the physiological state of growth, comparable to mitochondrial behavior in the basidiomycetous yeast *Bullera alba* (344). Similar

observations were made for the yeast-to-hyphae transition of *C. albicans* (13) of which only a single mitochondrion enters the emerging germ tube. It is possible that the differences observed between serial section analysis and fluorescence microscopy are due to differences in the *C. albicans* strains chosen for study. Differences may have also been observed due to experimental methods. For example, in the fluorescence studies an epifluorescence microscope was used to generate two-dimensional images of the mitochondria, whereas three-dimensional analysis was used in the serial-section studies. Confocal-scanning laser microscopy techniques have since been used to construct three-dimensional models of mitochondria in *S. cerevisiae* (354), but not in *C. albicans*.

The requirement of mitochondria for yeast cell viability, in conjunction with observed behavioral changes during cellular growth and differentiation has brought attention to the process of mitochondrial inheritance. Several studies have addressed the question of whether mitochondrial mobility patterns are linked to the cell cycle. Bud formation and germ tube formation are highly polarized processes in several fungal species (60,121) including *C. albicans* (84,132). Prior to bud formation, mitochondria localize to the cell cortex (153) and are transported to the emerging bud during the G_1-S phase of the cell cycle (333) suggesting that mitochondrial inheritance is tightly coordinated with the cell cycle. Both genetic and biochemical analyses suggest that this polarized transport of mitochondria to the bud is an active process that is actin-dependent (100,149,318). An intermediate filament-like protein, MDM1p has also been associated with mitochondrial inheritance (230,232). Temperature dependent MDM1p deficient mutants showed defects in transfer of mitochondria and nuclei into developing buds of

yeast cells at nonpermissive temperatures. Two additional actin-associated proteins, Mdm12p and Mdm20p are also required for mitochondrial inheritance (31,149). Yeast cells that failed to receive a mitochondrial compartment did not separate from the mother cell and were unable to produce buds (73,140,184,334). Further evidence that mitochondrial inheritance is linked to the cell cycle is provided by studies on a serine/threonine phosphatase, PTC1p in *S. cerevisiae* (286). Mutants deficient in *PTC1* displayed a delayed progression of mitochondrial inheritance. Changes in the actin cytoskeleton were not observed and nuclear migration appeared normal. Thus, the timing of mitochondrial transport also appears to be actively regulated and coordinated with the cell cycle.

Respiration in *C. albicans*

The biochemical properties of mitochondria as they relate to oxidative phosphorylation and respiration, and the elucidation of numerous metabolic pathways have been well characterized and extensively reviewed (139,348). In addition, the biochemical characterization of isolated *S. cerevisiae* mitochondria has been well-defined (382). The biochemical properties of mitochondria and respiration in *C. albicans* have been studied to some extent with preparations of crude mitochondrial extracts (259,377). *C. albicans* contains elements of the major respiratory enzyme complexes (245) such as NADH dehydrogenase (36,80), succinate dehydrogenase (245,377), cytochrome oxidase and electron transport chain subunits cytochromes b, c, a and aa3 (2,2,377).

The cytochrome pathway in fungi appears to be the major mode of electron transport (139,348). In addition to this classical cytochrome pathway, an alternative

pathway of electron transport has also been described in fungi (6). Electron transport via the cytochrome pathway is blocked by either cyanide, azide, or high levels of carbon monoxide. The alternative cyanide-insensitive respiration pathway is characterized by inhibition with either salicyl hydroxamate (SHAM) or azide. *C. albicans* possesses a cyanide-insensitive alternative respiratory pathway (11,199,200,357), which can be expressed in both yeast and hyphal cells (206,312). Accordingly, in the presence of cyanide, respiration of *C. albicans* cells is inhibited by SHAM (199,311). Studies on respiration deficient mutants in *C. albicans* indicates that the most likely branch point for the alternative pathway occurs between coenzyme Q and cytochrome b (11,199).

Growth conditions that favor the expression of the alternative oxidase in *C. albicans* remain undefined or are controversial. In one study, inhibition of electron transfer to complex IV by cyanide initiated the expression of the alternate oxidase in both yeast and hyphae (311). The initial addition of cyanide immediately reduced respiration in cells, but fully recovered to normal levels within 20 minutes. Subsequent addition of SHAM inhibited respiration. Other investigators, however, were only able to reproduce this phenomenon in aged yeast cells and not in hyphal cells or growing yeasts (10,11). Although the alternative pathway allows respiration to continue in the presence of cyanide, the cyanide-sensitive pathway is required for growth. The role of the alternate oxidase in the growth and development of *C. albicans* has not been determined.

Several lines of evidence indicate that there are differences in respiratory requirements between yeasts and hyphae. Early studies on morphogenesis indicated that respiratory activity in yeast cells declined upon addition to a hyphae-inducing medium

and continued throughout germination (375). In the presence of glucose, *C. albicans* hyphae have been shown to produce more ethanol, evolve less CO_2, and consume less oxygen than yeast cells, thus indicating an abrupt change from an aerobic to a fermentative metabolism (206).

Respiration Deficient Mutants

The generation of mitochondrial mutants in yeasts has contributed enormously to the understanding of the function of mitochondria, mitochondrial biogenesis, and genetic characterization of the mitochondrial genome. The original description of mitochondrial mutants came from studies on *S. cerevisiae* (94). Mutations in mitochondrial respiration, oxidative phosphorylation, and protein synthesis were induced by growth in the presence of acriflavine, which resulted in the occurrence of 'petite colonie' or cytoplasmic petite mutants. By definition, petites must be grown on a fermentable carbon source. On a medium containing 0.1% glucose and 2% glycerol, wild-type cells form large colonies, whereas the colonies formed by petite mutants are small (118). Petite mutant cells use glucose as the carbon source and they are incapable of respiring the glycerol. Further studies on acriflavine-induced mutants have demonstrated that the observed mitochondrial deficiencies result from large deletions of the mitochondrial genome (97). In addition to nucleic acid mutations, acriflavine inhibits the synthesis of cytochrome components of the respiratory system during aerobic growth, which most likely precedes mutagenesis (47). Acriflavine-induced cytochrome inhibition is the result of blockage in mitochondrial protein synthesis and is similar in mechanism to chloramphenicol (188).

Growth of *S. cerevisiae* petite mutants is supported by fermentation in the absence of a functional mitochondrial oxidative phosphorylation system. (348).

Numerous attempts have been made to generate petite mutants in *C. albicans*. *C. albicans* was described as a petite-negative yeast in 1964 (47). Since then, acriflavine-induced petite mutants have been described (370). These *C. albicans* repiration-deficient mutants approximate *S. cerevisiae* cytoplasmic petites, namely reduced oxygen consumption and growth rates. Several *C. albicans* petite mutants also demonstrated the loss of cytochrome aa3 (12), which is a characteristic of *S. cerevisiae* petites (118). It is clear that these *C. albicans* acriflavine-induced mutants share features of *S. cerevisiae* petites and have impaired mitochondrial function. However, their classification as petite mutants is controversial. Others have reported *C. albicans* respiration deficient mutants, but had difficulties in obtaining petites (2).

The rigorous genetic screening used to define mitochondrial DNA deletions in *S. cerevisiae* cytoplasmic petite mutants was not used to define the *C. albicans* petite mutants. For example, specific mutational events in the *C. albicans* mitochondrial genome were not documented (370) and respiration deficiency in these mutants most likely stems from lesions in nuclear genes (284). Several nuclear defects in *S. cerevisiae* are associated with mitochondrial dysfunction (97). Cytoplasmic petites in *S. cerevisiae* also display a loss of functional cytrochromes aa_3, b, and c (107), whereas the *C. albicans* petite mutants were only deficient in cytrochrome aa_3 (376) or diplayed the complete cytochrome spectrum (12). *C. albicans* respiration deficient mutants not referred to as petite mutants (reduced oxygen consumption and growth rate) were also deficient in

cytochrome aa_3 and additionally lacked cytochrome b (2). Furthermore, *S. cerevisiae* mitochondrial petite mutants are classified into vegetative (non-mendelian) or segregational (mendelian) petites based on mitochondrial DNA inheritance patterns (118), but this kind of analysis was not done on the *C. albicans* mutants (12). Based on the original description of petite mutants in *S. cerevisiae* (94), *C. albicans* appears to be a petite negative yeast.

Mitochondrial Genetics

An interesting feature of mitochondria is the requirement of an independently replicating genome in mitochondria of some eukaryotes, and the complete absence of a mitochondrial genome in others (118). This feature raises questions concerning the necessity for, and maintenance of a mitochondrial genome. The mitochondrial genome contains genes for several essential mitochondrial proteins, but the nuclear genome of the majority of eukaryotes contains genes for over 90% of the mitochondrial proteins (348). In fact, indirect evidence suggests that organisms are evolving away from the requirement of a mitochondrial genome. Complete loss of mitochondrial DNA has occurred in several eukaryotes, but these organisms retain an energy generating mitochondrial-like organelle called a hydrogenosome (32,265). Similar to mitochondria, hydrogenosomes have a double-membrane envelope, divide autonomously by fission, import proteins post-translationally, and can produce ATP by substrate-level phosphorylation (83). In organisms with hydrogenosomes, genes encoding three mitochondrial heat shock proteins (Hsp10, Hsp60, Hsp70) are found on nuclear chromosomes (46), which suggests a mitochondrial origin. Hydrogenosomes are

characteristic to several anaerobic eukaryotes including many ciliates and protists (265). The hydrogenosomes in these organisms resemble mitochondria of anaerobically grown *S. cerevisiae*. Anaerobic growth of *S. cerevisiae* on glucose causes a reduction in respiratory cytochromes and prevents the formation of mitochondrial unsaturated fatty acids and ergosterol (266). The mitochondria of these *S. cerevisiae* lipid-depleted cells are inactive in protein synthesis, lack mitochondrial DNA, and are incapable of converting to aerobic respiration (118).

Mechanisms for the retention and requirement of mitochondrial DNA in the majority of eukaryotes have not been well defined. The hydrophobic nature of several mitochondrial proteins may well necessitate an intra-organellar site of synthesis, thus obviating a need for transport from the aqueous environment of the cytoplasm to the mitochondrion (66,67). A prediction is that extremely hydrophobic proteins required for mitochondrial function must be encoded internally in the mitochondrial genome (138). The problem with this hypothesis is that the highly hydrophobic ATPase subunit 9 is encoded by a nuclear gene in *Neurospora crassa* and is transported to the mitochondria (59), which requires transport across the aqueous cytoplasm before assembly in the mitochondrial inner membrane. This indicates that hydrophobicity of mitochondrial encoded proteins is not the only requirement for the retention of a mitochondrial genome.

Mitochondrial Genome Organization and Gene Content

The relative importance of mitochondrial DNA has led to considerable efforts to sequence entire mitochondrial genomes in several species including humans (8).

Mitochondrial sequence analysis in humans has uncovered numerous genetic disorders resulting from deficiencies or mutations in mitochondrial genes (207,380). The fact that mitochondrial DNA has much less redundancy than the nuclear genome and much higher information density (an equivalent complement of essential nuclear genes would span 1-2 million base pairs), makes it an excellent target for the expression of mutants. However, not all mitochondrial mutants are viable, which has led to interest in identification of mitochondrial genes.

Several yeast mitochondrial genomes have been sequenced in their entirety including: *Hansenula wingeii, S. douglasii, Schizosaccharomyces pombe, S. cerevisiae,* and *S. uuvarum* (53,54,82,267,308). This list is not exhaustive and efforts are currently in progress to obtain representative mitochondrial genome sequences across the entire fungal taxa (267). Analysis of mitochondrial DNA sequences has revealed several genes ubiquitous to fungi. The majority of fungal mitochondrial DNA contains genes encoding large and small ribosomal RNAs, a complete spectrum of tRNAs, three subunits of cytochrome c oxidase, three subunits of ATPase, and apocytochrome b (119). Differences have been observed between yeasts and filamentous fungi. Mitochondrial DNA in several yeast species contains the VAR1 gene, encoding a mitochondrial ribosomal protein (82,119,308). Filamentous fungi generally contain mitochondrial genes encoding subunits of NADH dehydrogenase (44,82,308). NADH dehydrogenase genes are an unusual component of the yeast *H. wingeii*, and are not found in *S. cerevisiae* (308). Size differences in fungal mitochondria are attributed to the presence/absence of introns and to the length of intergenic regions (82,371). Introns in *S.*

cerevisiae contain short G + C rich sequences (GC clusters), which are mobile elements and hot spots for recombination in the mitochondrial genome (359). In contrast to vertebrate mitochondrial genomes, which generally have G+C contents greater than 40%, the G+C content of most yeast species is around 18% (82), which indicates that G+C composition may be of regulatory importance in yeasts.

The structural organization of genes in mitochondrial genomes is also highly variable (371). One interesting structural feature of these genomes is the presence of amphimers or inverted repeats. Inverted repeat sequences are common in chloroplast genomes, but are unusual in mitochondrial DNA (275). In fungi, inverted repeats have been reported in *Achlya*, *Phythium*, *Agaricus*, *Agrocybe*, *C. albicans*, and *Kloeckera africana* (64,151,162,236,368). Special cases of multiple inverted repeats have been noted in the mitochondria of petite mutants in *S. cerevisiae*, but not in their wild-type parent strains (276). The function of inverted repeats in mitochondrial DNA is unclear, but others have proposed that the structure of inverted repeats may be the only form in which a duplicated segment can be stably retained in an amphimeric genome (37). If duplicated segments of DNA are present as tandem repeats in an amphimeric genome, homologous recombination would tend to eliminate one copy (275).

The majority of mitochondrial genomes appear to be circular (26), but linear forms are also known to exist in fungi (108). The mechanism for mitochondrial replication is under investigation, and the rolling circle method may occur in yeasts (223). Linear mitochondrial DNA molecules may be intermediates of replication (17,26,27) rather than an artifact due to mechanical shearing during DNA isolation (26),

but this point has not been resolved. In many cases, the linear form of mtDNA is larger than the circular forms, which would tend to rule out a broken circle theory (26). Furthermore, several fungi contain linear mitochondrial DNA molecules with hairpin structures at both termini, suggesting that these structures are present to preserve the ends of the linear genome (86). Pulsed-field electrophoretic methods often reveal the presence of both linear and circular forms of mitochondrial DNA (26), which indicates that both forms are important in both replication and function (17,27).

Mitochondrial genomes are organized into electron dense areas referred to as nucleoids or nuclei (203). Within nucleoids, mitochondrial DNA is associated with arginine/lyisine- rich proteins, which resembles nuclear histones (55). The number of mitochondrial genomes per nucleoid varies between one and eight (371), but most mitochondria contain one nucleoid (203). Mitochondrial genome copy number per cell is also variable. The *S. cerevisiae* mitochondrial genome copy number is approximately 50 per haploid cell, which is based on the amount of mitochondrial DNA relative to total DNA (13.5±1.3%) (140). However, the amount of mitochondrial DNA per cell has been reported to vary between 5 and 25% of total DNA (371). By extrapolation, the number of mitochondrial genomes in *S. cerevisiae* may vary between 17 and 87.

The timing of mitochondrial replication in relation to the cell cycle appears to be species dependent. In *S. cerevisiae*, replication of mitochondrial DNA takes place continuously throughout the cell cycle (202,241,366). In contrast, mitochondrial DNA synthesis is periodic during synchronous growth in *S. pombe* and *S. lactis*, and occurs at a different time than nuclear DNA replication (79,323). The entire mitochondrial DNA

population may replicate synchronously in an *S. cerevisiae* culture, providing further evidence of nuclear control of mitochondrial DNA replication in this fungus (70).

Mitochondrial DNA in *C. albicans*

The *C. albicans* mitochondrial genome is circular with a molecular size of 40 kb (309,367,368). The base composition of the genome consists of a G+C content of 38.2%, which is similar to the 33% G+C content of the nuclear genome (368). The G+C content is exceedingly higher than the 18% G+C content of *S. cerevisiae* mitochondrial DNA and more similar to the 40% G+C content of vertebrates (82). Extensive restriction map analysis of *C. albicans* mitochondrial DNA has been done by use of an *EcoR*I library of the circular genome (368). The use of the *EcoR*I fragments as DNA probes, revealed the presence of a large inverted repeat in the mitochondrial genome (309,368). The unique feature of the inverted repeat is that it does not contain rDNA, which is a characteristic of the majority of mitochondria and chloroplasts containing inverted repeats (275). Hybridization analysis using *S. cerevisiae* mitochondrial gene probes indicates that the inverted repeat contains the subunit 3 of cytochrome oxidase (309). However, the gene content of the majority of the inverted repeat remains unknown. The map position of other *C. albicans* mitochondrial genes was also determined by using known *S. cerevisiae* genes as probes (see figure 1) (309). The sequence of several mitochondrial genes in *C. albicans* has been revealed by efforts towards sequencing the entire genome, but their position on the mitochondrial map has not been determined.